Reference: *Biol. Bull.* 172: 122–127. (February, 1987)

RECENT DEVELOPMENTS IN THE MOLECULAR STRUCTURE OF CIGUATOXIN

KAZUO TACHIBANA, MANABU NUKINA, YONG-GOE JOH, AND PAUL J. SCHEUER

Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822

Abstract

Crystalline ciguatoxin isolated from moray eel (*Lycodontis* ≡ *Gymnothorax javanicus*) viscera has an LD_{50} of 0.45 g/kg (i.p., mice). It has a molecular weight of 1111.7 ± 0.2 daltons. ^1H NMR studies have shown that it is a polar and highly oxygenated molecule belonging to the class of polyethers. On basic alumina ciguatoxin is reversibly converted to a chromatographically distinct less polar form, which is equally toxic and elicits typical ciguatoxin symptoms in mice.

From parrotfish (*Scarus sordidus*), which originated on a ciguateric reef on Tarawa atoll (Kiribati), we have isolated two toxins that evoke ciguatera symptoms in mice at approximately equal levels. Chromatographic evidence suggests that the two toxins are identical with the two ciguatoxins of different polarity and that the less polar form is the previously described scaritoxin.

Introduction

> "He who oversees everything also created very many poisonous fish, in this way he punishes those who seek them."
>
> —*J. Grevin*

In his book on venoms the 16th century, French physician and poet Jacques Grevin (Grevin, 1568) intuitively foresaw some of the frustration which has been the hallmark of ciguatera research during the past thirty years. Although ciguatoxin, the principal toxin in ciguateric carnivorous fish, may be superficially compared with the well-known marine toxins tetrodotoxin and the saxi-gonyautoxins, they share few characteristics with ciguatoxin. The single factor responsible for the slow progress in ciguatera research is that ciguatoxin is a slow-acting toxin which is rarely fatal to man. It is rarely fatal not because of its lack of potency—indeed its potency is surpassed only by that of palytoxin (0.15 μg/kg; Moore and Scheuer, 1971) and maitotoxin (0.2 μg/kg; Ohizumi and Yasumoto, 1983)—consumers of ciguateric fish survive because of its extremely low concentration in fish flesh. Thus, it has been difficult to accumulate enough toxin for structural research and—for many years—to convince skeptical fellow scientists of the very existence of a well-defined toxic entity. As a slowly acting toxin, ciguatoxin lacks the dramatic impact of a fast-acting toxin, somehow a convincing demonstration of the power of a toxin.

Methods and Results

Because of the many variables attending ciguatera outbreaks, among them place, time, species of fish, and reported symptoms in man, we have confined our research to toxin isolated from the moray eel (*Lycodontis* ≡ *Gymnothorax javanicus*), initially from flesh and viscera, but more recently exclusively from viscera in order to economize on solvents and shipping cost. Geographically, the eels originated from John-

FIGURE 1. Ciguatoxin extraction.

ston atoll (165°W, 17°N) and more recently from Tarawa atoll (173°E, 1°30′N), Republic of Kiribati.

Figures 1 and 2 summarize our procedure for toxin extraction and purification (Tachibana, 1980). Approximately 75 kg of eel viscera, representing some 1100 kg of toxic eels, yielded 1.3 mg of chromatographically pure toxin. The final HPLC trace (Fig. 3) obtained on a C_{18} reversed phase silica column is a symmetrical peak. The toxin, LD_{50} 0.45 μg/kg (i.p., mice), is a colorless solid readily soluble in methanol, ethanol, 2-propanol, and acetone, sparingly soluble in chloroform and diethyl ether, and nearly insoluble in water or benzene.

Ciguatoxin displays a single UV absorption peak at 215 nm (ϵ 5250). At that wavelength its CD (circular dichroism) molecular ellipticity is -620. The most prominent features in the infrared spectrum (FT, solid film) of ciguatoxin are hydroxyl (3450 cm^{-1}) and ether (1080 cm^{-1}) bands. A respectable signal at 1600 cm^{-1} cannot be unequivocally interpreted.

Californium-252 plasma desorption mass spectrometry pointed to a likely molecular weight of 1111.7 ± 0.3 daltons (R. D. Macfarlane and C. McNeil, pers. comm.). Since this technique is unsuitable for high resolution measurements, no molecular formula of ciguatoxin was obtained. On the basis of ^1H NMR data, formulas such as $C_{53}H_{77}NO_{24}$ (1112.2) or $C_{54}H_{78}O_{24}$ (1111.2) are reasonable.

The bulk of the structural information was derived from extensive ^1H NMR experiments at 360 and 600 MHz in methanol-d_4 or dimethylsulfoxide-d_6. Methanol gives rise to a well-defined spectrum, but provides no clues for exchangeable protons. Ciguatoxin possesses five hydroxyl groups, four carbon-carbon double bonds, and five methyls, all on saturated carbon. The combined structural pieces account for

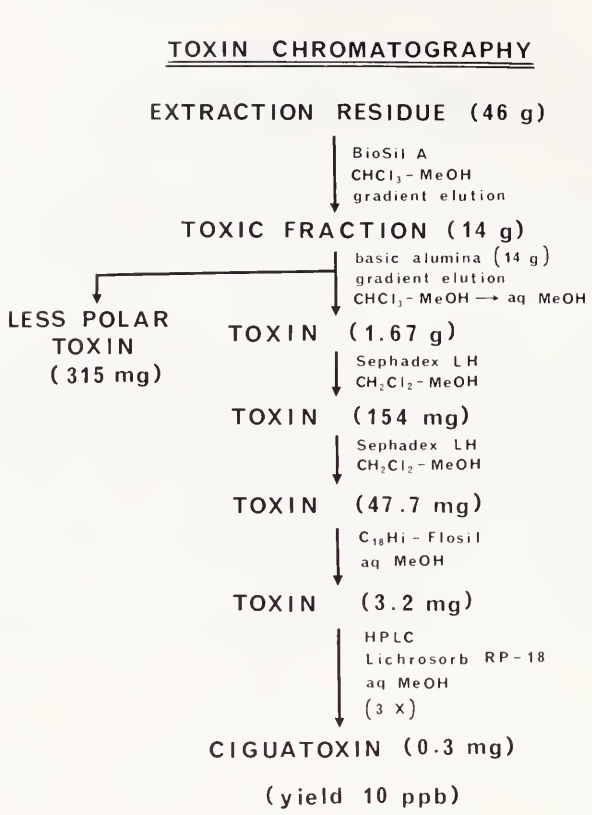

FIGURE 2. Ciguatoxin purification.

nearly 75 hydrogen atoms and imply 54 carbon atoms, but in the absence of a carbon spectrum it is impossible to judge the degree of overlap, which casts doubt on the reliability of the carbon count. Although no satisfactory carbon-13 spectrum of ciguatoxin has been determined because of lack of toxin and/or sufficient instrument time, our sample crystallized in an NMR tube during an attempt to have the carbon spectrum measured at a mainland applications laboratory. The crystals, unfortunately, are too small to be suitable for x-ray diffraction studies, and our attempts at growing larger crystals have so far failed.

DISCUSSION AND CONCLUSION

When one considers the broad spectrum of symptoms which ciguatera-intoxicated patients describe and the difficulty of isolating a homogeneous toxin from a complex matrix in which it is present at a concentration of approximately $1 \times 10^{-6}\%$, the question of multiple toxins inevitably arises. In the absence of convincing evidence to the contrary, we have followed the simplest assumption in our research; *i.e.*, we have assumed that ciguatoxin is a single entity. Yet occasionally we observed (Tachibana, 1980) that in samples of extracts that had been stored for some time, ciguatoxin would be eluted from a silica column with a less polar solvent mixture (chloroform/methanol 97:3) than is the case normally, when the bulk of the toxin is eluted with a 9:1 chloroform/methanol solution. The existence of a less polar form

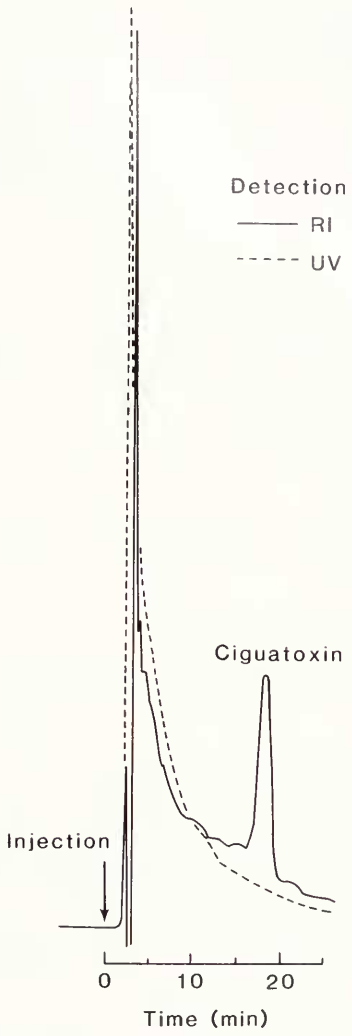

FIGURE 3. Reversed phase HPLC of ciguatoxin.

of ciguatoxin can be demonstrated by chromatography on basic alumina of different activity grades. We showed (Nukina *et al.*, 1984) that the two forms of ciguatoxin, while chromatographically distinct, are interconvertible. The two forms have ^{1}H NMR spectra which differ only in minor details and elicit comparable symptoms in mice.

An epidemiological survey including detailed case studies in the Gambier islands, where ciguatera intoxication arises principally from eating parrotfishes (Scaridae), led Bagnis *et al.* (1974) to suggest that either a new toxin or multiple toxins were involved. In her follow-up, Chungue (1977; Chungue *et al.*, 1977) isolated from the flesh of *Scarus gibbus* a toxic mixture which was separable by DEAE cellulose chromatography into a toxin designated scaritoxin and a more polar toxin which strongly resem-

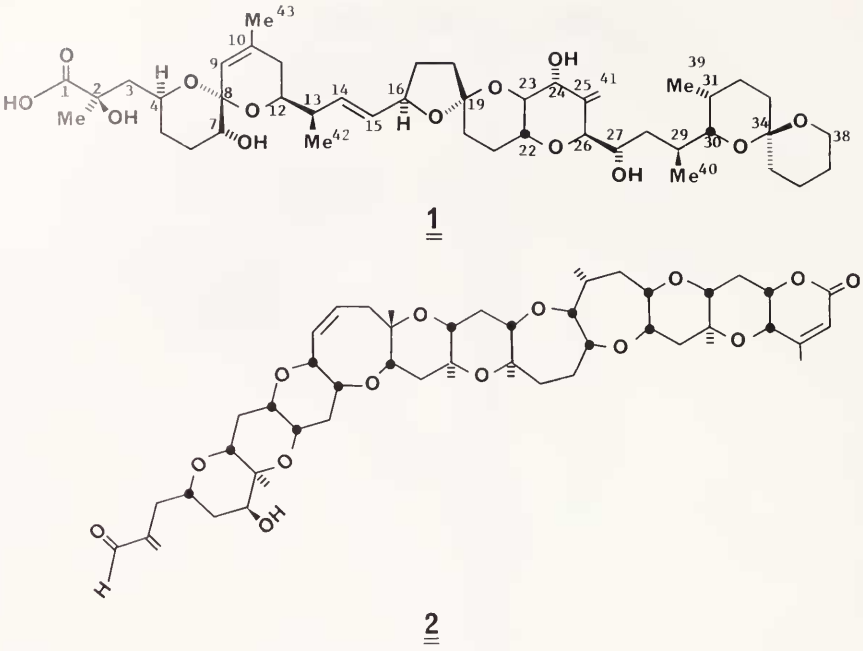

FIGURE 4. (1) Molecular structure of okadaic acid. (2) Molecular structure of brevetoxin B.

bled ciguatoxin. Scaritoxin was reported to cause severe hind limb paralysis in mice, a symptom not normally observed with ciguatoxin.

Recently, we (Joh and Scheuer, in press) examined parrotfish (*Scarus sordidus*) from a toxic reef on Tarawa atoll, Republic of Kiribati. We also isolated two toxins separable on DEAE cellulose. However, by manipulation on basic alumina we were able to interconvert the two toxins. By TLC comparison we showed that scaritoxin and the less polar ciguatoxin (Nukina *et al.*, 1984) are identical, though this finding remains to be confirmed by spectral comparison.

When Yasumoto (Murakami *et al.*, 1982) examined the constituents of the toxic dinoflagellate *Prorocentrum lima,* he made the surprising discovery that on TLC analysis one of the constituents was indistinguishable from ciguatoxin. The substance proved not to be ciguatoxin but okadaic acid (Fig. 4, 1), a compound which we had shortly before reported from a sponge, *Halichondria okadai* (Tachibana *et al.*, 1981). The two compounds differ greatly in size (1111 *vs.* 804 daltons) and lethality (0.45 *vs.* 192 μg/kg), but evidently not in polarity because of their similar chromatographic behavior. This was the first clear indication that ciguatoxin belongs to the class of polyethers, as does, *inter alia,* brevetoxin B (Fig. 4, 2) (Lin *et al.*, 1981). These compounds are highly oxygenated long-chain fatty acids, in which most of the oxygen atoms occur as cyclic ether linkages. Okadaic acid (Fig. 4, 1), possesses one carboxyl, four hydroxyls, and seven oxa rings, in addition to three carbon-carbon double bonds. This information allows us to extrapolate safely to the ciguatoxin structure, which evidently is a complex polyether possessing five hydroxyls and four carbon-carbon double bonds. This close structural analogy to okadaic acid (Fig. 4, 1) and to brevetoxin (Fig. 4, 2) provides a plausible rationale for the interchangeability of different chromatographic forms of ciguatoxin and scaritoxin. The presence in cigua-

toxin of multiple hydroxyl groups permits formation and destruction of various hydrogen-bonded forms while preserving the structural integrity of the molecule. Because of its large size, ciguatoxin may well assume two or more secondary shapes which prevail under different conditions of basicity.

Perhaps the most intriguing questions posed upon examination of these compounds are those dealing with their mechanism of physiological action and with the subtle structural features that give rise to profound differences in lethality and overt symptoms in mammals.

ACKNOWLEDGMENT

We thank the National Marine Fisheries Service (NA80AA-00101), through a subcontract with the Medical University of South Carolina, for financial support.

LITERATURE CITED

BAGNIS, R., E. LOUSSAN, AND S. THEVENIN. 1974. Les intoxications par poissons perroquets aux îles Gambier. *Med. Trop.* **34:** 523–527.

CHUNGUE, E. 1977. *Le Complexe Toxinique des Poissons Perroquets.* Ph.D. Thesis, Academie de Montpelier, Université des sciences et techniques du Languedoc, Montpelier, France.

CHUNGUE, E., R. BAGNIS, N. FUSETANI, AND Y. YASHIMOTO. 1977. Isolation of two toxins from a parrotfish *Scarus gibbus. Toxicon* **15:** 89–93.

GREVIN, J. 1568. *Deux Livres des Venins.* Christofle Plantin, Antwerp, Belgium.

JOH, Y.-G., AND P. J. SCHEUER. In press. The chemical nature of scaritoxin. *Marine Fish. Rev.*

LIN, Y. Y., M. RISK, S. M. RAY, J. CLARDY, D. VAN ENGEN, J. GOLIK, J. C. JAMES, AND K. NAKANISHI. 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). *J. Am. Chem. Soc.* **103:** 6773–6775.

MOORE, R. E., AND P. J. SCHEUER. 1971. Palytoxin: a new marine toxin from a coelenterate. *Science* **172:** 495–498.

MURAKAMI, Y., Y. OSHIMA, AND T. YASUMOTO. 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima. Nippon Suisan Gakkaishi* **48:** 69–72.

NUKINA, M., L. M. KOYANAGI, AND P. J. SCHEUER. 1984. Two interchangeable forms of ciguatoxin. *Toxicon* **22:** 169–176.

OHIZUMI, Y., AND T. YASUMOTO. 1983. Contraction and increase in tissue calcium content induced by maitotoxin, the most potent known, marine toxin, in intestinal smooth muscle. *Br. J. Pharmacol.* **79:** 003–005.

TACHIBANA, K. 1980. *Structural Studies on Marine Toxins.* Ph.D. Thesis, University of Hawaii, Honolulu.

TACHIBANA, K., P. J. SCHEUER, Y. TSUKITANI, H. KIKUCHI, D. VAN ENGEN, J. CLARDY, Y. GOPICHAND, AND F. J. SCHMITZ. 1981. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria. J. Am. Chem. Soc.* **103:** 2469–2471.